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Studies on Proteins. VIII.

The Influence of Salts on The Iso-electric Behavior of The Protein

By

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With 7 tables and 12 text figures

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A. Introduction

The point of maximum flocculation of such a difficult soluble protein as rice-glutelin or casein does not show the iso-electric point of the protein. We call this point an apparent iso-electric point.(1) This apparent iso-electric point is moveable according to the concentration and kind of salts in the buffer solution. This is caused by the fact that the protein in the salt solution is not able to behave as an iso-electric protein, because the addition of salt causes a change in the hydrogen ion activity of the solution, and the cations and anions, which are dissociated from the salt, force the protein molecule to ionize on the opposite charge and in a different degree. Hence, the protein molecule will be ionized according to the differences among the protein ionizeing powers of the ions derived from the salt. Therefore, such a protein as casein or rice-glutelin under the presence of salts, can not

(1) Kondo K. and Hayashi T., Memoirs of Coll. Agri., Kyoto Imp. Univ. No. 2. Art. 2. (1926).

take an iso-electric form, even in the iso-electric solution. E. J. Cohn[1] has already claimed that the presence of the foreign protein (with different solubility and different iso-electric point), of multivalent ions, or of neutral salts may shift the point of minimum solubility of the protein; hence the presence of these impurities renders impossible the determination of the true iso-electric point. This claim may be reasonable from one point of view.

Moreover, if the concentration of the salt increase, the protein molecules may combine with one component of the salt and form a complex-compound. This complex-compound may be soluble or insoluble according to the concentration of the salt. Hence, the protein may not flocculate in a simple iso-electric form at the point of maximum flocculation in such a salt solution. These influences of salts on the iso-electric behavior of the protein are variable according to the kinds of salts and their concentrations. The present work has for its purpose a more distinct elucidation of the above phenomena according to experimental results.

B. Experimental Results. (1)

The protein, studied in this work, is Rice-glutelin No. 2. The method[2] of preparation of this protein has been already described. The purified and dialysed rice-glutelin is washed again with water by means of a centrifugal machine and subsequently treated with absolute alcohol and ether. The glutelin is then made free from alcohol and ether for several days in an incubator, which is regulated electrically at 40°C. After this procedure we reserved the glutelin in a sulphuric acid dessicator.

With this protein we prepare the stock solution in the following manner. 10 g.[3] of the rice-glutelin is mixed with ca. 400 cc. of pure water in a 1 L. measuring flask and then it is put into an ice-box, with an occasional shaking to make the water penetrate the protein particles. After one night, 100 cc. of 0.1 n NaOH previously diluted with ca. 300 cc. of pure water, is dropped into the flask with continu-

(1) Cohn, E.J., Gen. Physiol., **4**, No. 6. 697–722 (1922).

(2) Kondo K. and Hayashi T., Memoirs of Coll. Agri., Kyoto Imp. Univ. No. 2. Art. 1. (1926).

(3) After a preliminary experiment we found that 10 g. of the protein is suitable for 1 L. of solution containing 100cc. of 0.1 n NaOH.

ous shaking and the flask is filled up with water. Then the flask containing the protein is immersed in a water-bath at 18°C and occasionally shaken in order to dissolve the protein thoroughly. After 24 hours, we see the solution saturated with protein and a certain portion of the protein which could not be dissolved at the bottom of the flask. The flask is then filled up to the mark in the water-bath at 15°C. After this procedure, the solution is kept at 18°C and filtered through a Buffner funnel to avoid there remaining any undissolved portion of the rice-glutelin. The funnel is fitted in layers with a purified filter pulp, previously washed with water, and with a 0.01 n NaOH solution. The filtrate is reserved in a colored bottle, which is surrounded by pieces of ice in an ice-box.

Experiment a.

After the experiments described in the preceding report[1] we know that Rice-glutelin No. 2 can flocculate at the optimum at paH 5.38–5.08 in a 0.1 n sodium acetate solution, which is 0.02–0.04 n in respect to acetic acid. Therefore, we have now to study the influence of salts on the rice-glutelin in such an apparent iso-electric solution as described above.

First we experimented with the alkaline chlorides, e.g. KCl, NaCl, RbCl and LiCl, and summarized the results in Table 2. In each experiment 25 cc. of the stock solution were mixed with a calculated quantity of 0.5 n acetic acid, of 0.5 n sodium acetate, of the salt studied and of water in the manner described in Table 2. The flocculated rice-glutelin is kjeldahled and the paH-value of the filtrate is determined by our usual method. In Table 1 we show for reference the amounts of various solutions used in the experiment shown in Table 2.a.

Table 1.

Experiment No.	Stock solution	Na-CH_3COO 0.5 n	HCH_3COO 0.5 n	RbCl	H_2O
	cc.	cc.	cc.	g.	cc.
1	25	9.5	2.5	6.0500	to 50
2	25	9.5	2.5	4.5375	to 50
3	25	9.5	2.5	(2n) 12.5 cc	to 50
4	25	9.5	2.5	(n) 10.0 cc	to 50
5	25	9.5	2.5	(n) 2.5 cc	to 50
6	25	9.5	2.5	(n) 0.5 cc	to 50

(1) Kondo K, Hayashi T, Matsushita T., Memoirs of Coll. Agri., Kyoto Imp. Univ. No. 2. Art 3. (1926),

Table 2. a.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{RbCl} n	Protein-N Precipitated mg.	paH
1	40.94	0.1	0.02	1.0	40.85	5.251
2	,,	,,	,,	0.75	40.79	5.270
3	,,	,,	,,	0.5	40.53	5.281
4	,,	,,	,,	0.2	40.53	5.286
5	,,	,,	,,	0.05	40.66	5.314
6	,,	,,	,,	0.01	40.79	5.317
7	,,	,,	,,	0.00	40.68	5.335

Table 2. b.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{KCl} n	Protein-N Precipitated mg.	paH
1	40.94	0.1	0.02	1.0	40.06	5.243
2	,,	,,	,,	0.75	40.04	5.260
3	,,	,,	,,	0.5	39.78	5.273
4	,,	,,	,,	0.1	40.56	5.295
5	,,	,,	,,	0.05	40.66	5.303
6	,,	,,	,,	0.01	40.72	5.317

Table 2. c.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{NaCl} n	Protein-N Precipitated mg.	paH
1	40.94	0.1	0.02	1.0	39.67	5.208
2	,,	,,	,,	0.75	39.23	5.224
3	,,	,,	,,	0.5	39.36	5.250
4	,,	,,	,,	0.2	39.88	5.292
5	,,	,,	,,	0.05	40.53	5.334
6	,,	,,	,,	0.01	40.79	5.339

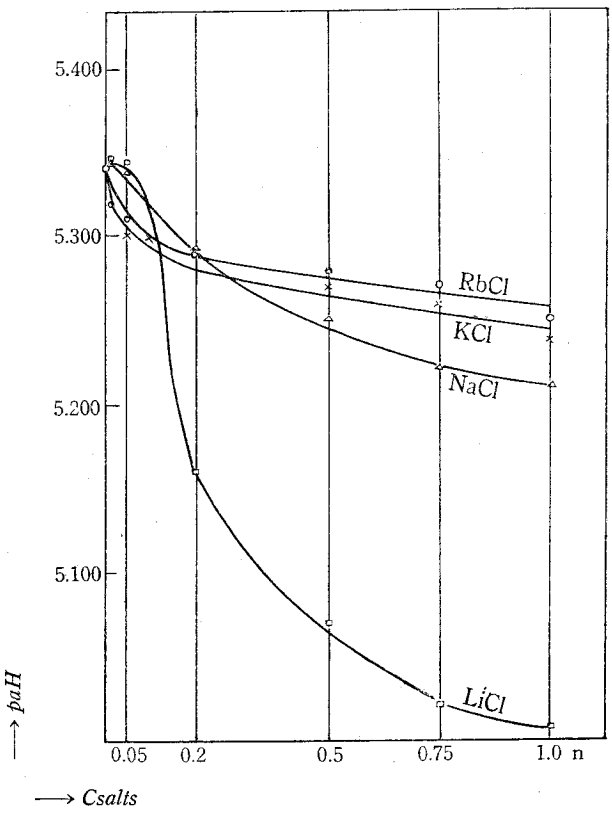
As shown in Table 2 and Figure 1 the addition of the salt to the solution, the reaction of which is apparently iso-electric for the rice-glutelin, shifts the reaction of the solution to the acidic side, and the

Table 2. d.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{LiCl} n	Protein-N Precipitated mg.	paH
1	40.94	0.1	0.02	1.0	39.66	5.006
2	,,	,,	,,	0.75	39.20	5.023
3	,,	,,	,,	0.5	39.28	5.068
4	,,	,,	,,	0 2	39.93	5.191
5	,,	,,	,,	0.05	40.46	5.336
6	,,	,,	,,	0.01	40.64	5.341

higher the concentration (0.01–1.0 n) of the salt becomes, the more this shift of the reaction. Moreover, the degree of shifting differs

Fig. 1.



according to the kind of salt. We find the following order. RbCl< KCl<NaCl<LiCl. Above all, the influence of LiCl is very distinct. Therefore, with chlorides the hydrogen ion activity of the solution changes in the order $Li^{\cdot}>Na^{\cdot}>K^{\cdot}>Rb^{\cdot}$.

These chlorides not only shift the reaction of the solution, but also influence the flocculation of the rice-glutelin, as we see in Table 2 and Figure 2, and the higher the concentration of the salt becomes, for RbCl and KCl in the concentration 0.01–0.5 n and for NaCl and LiCl 0.01–0.75 n, the less the amount of precipitated rice-glutelin. But, if the salt concentration increase to more than this, the amount of flocculated protein will again increase. These influences of the salts or cations on the precipitability of the protein parallel these influences on the hydrogen ion activity of the solution.

Fig. 2.

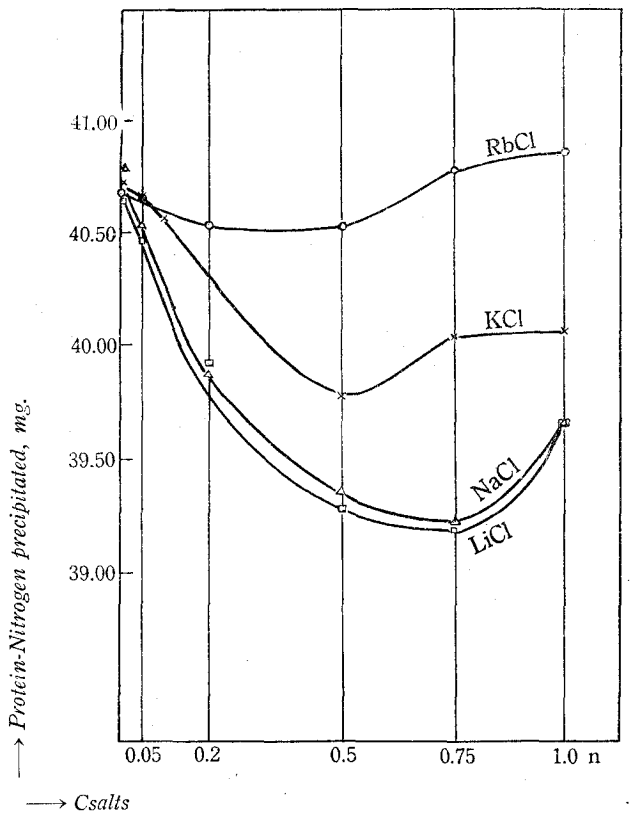


Table 3. a.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{BaCl_2} n	Protein-N Precipitated mg.	paH
1	41.01	0.1	0.02	0.1	40.12	5.289
2	,,	,,	,,	0.05	40.80	5.331
3	,,	,,	,,	0.02	40.49	5.353
4	,,	,,	,,	0.01	40.59	5.362
5	,,	,,	,,	0.005	40.59	5.358
6	,,	,,	,,	0.000	40.54	5.379

Table 3. b.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{CaCl_2} n	Protein-N Precipitated mg.	paH
1	41.01	0.1	0.02	0.1	40.07	5.284
2	,,	,,	,,	0.05	40.59	5.316
3	,,	,,	,,	0.02	40.75	5.345
4	,,	,,	,,	0.01	40.45	5.364
5	,,	,,	,,	0.005	40.33	5.372

Table 3. c.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{MgCl_2} n	Protein-N Precipitated mg.	paH
1	41.01	0.1	0.02	0.1	40.02	5.270
2	,,	,,	,,	0.05	40.43	5.314
3	,,	,,	,,	0.02	40.64	5.335
4	,,	,,	,,	0.01	40.33	5.355
5	,,	,,	,,	0.005	40.23	5.355

Table 3. d.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{CoCl_2} n	Protein-N Precipitated mg.	paH
1	41.01	0.1	0.02	0.02	40.28	5.331
2	,,	,,	,,	0.01	40.43	5.346
3	,,	,,	,,	0.005	40.28	5.346

Table 3. e.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{NiCl_2} n	Protein-N Precipitated mg.	paH
1	41.01	0.1	0.02	0.02	40.64	5.300
2	,,	,,	,,	0.01	40.75	5.314
3	,,	,,	,,	0.005	39.70	5.359

Table 3. f

No.	Total protein-N in Solution mg.	$C_{\frac{NaCH_3COO}{n}}$	$C_{\frac{HCH_3COO}{n}}$	$C_{\frac{CuCl_2}{n}}$	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.1	40.12	4.902
2	,,	,,	,,	0.05	40.43	5.143
3	,,	,,	,,	0.02	40.73	5.274
4	,,	,,	,,	0.01	40.80	5.316
5	,,	,,	,,	0.005	40.70	5.302

Fig. 3. a.

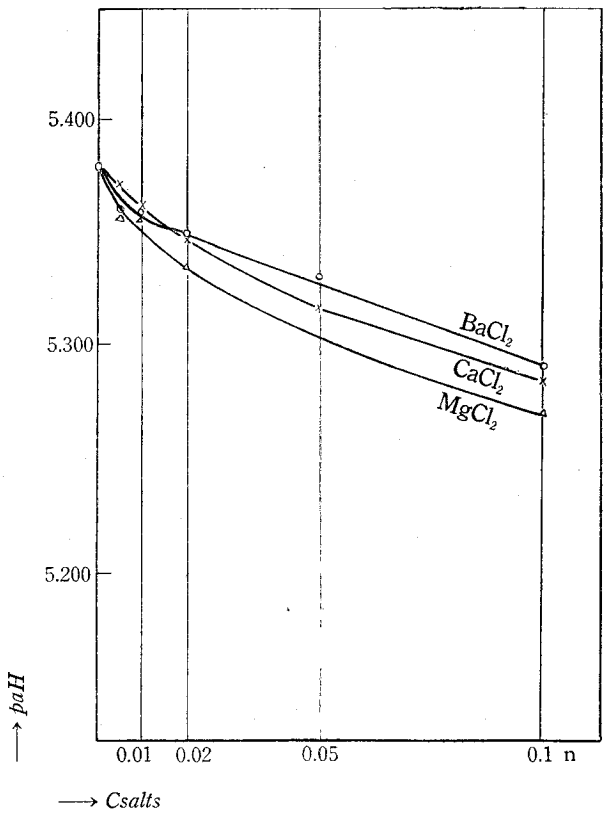
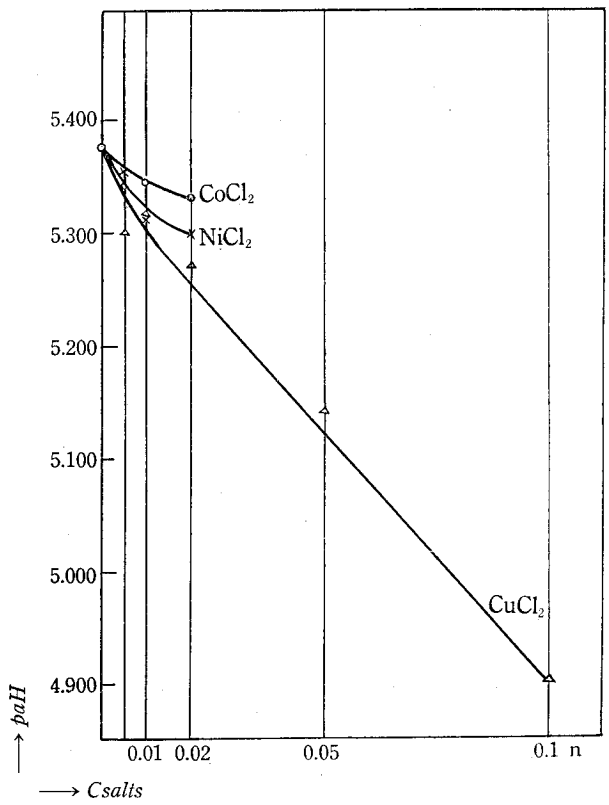


Fig. 3. b.



Experiment b.

In this experiment we examined the influence of the bivalent cations on the iso-electric behavior of the rice-glutelin. The salts used were $BaCl_2$, $CaCl_2$, $MgCl_2$, $CoCl_2$ and $NiCl_2$. The plan of the experiment was the same as that of the preceding experiment.

After Michaelis,[1] in experiments with chlorides we note a stronger result with bivalent cations than with univalent cations. Hence we used a more diluted salt solution in this experiment than in that of the preceding. The results are summarized in Table 3 and Figures 3 and 4.

These results show that the addition of these salts to the solution, the reaction of which is apparently iso-electric for the rice-glutelin, shifts

(1) Michaelis L.u. von Szent-Gyorgyi; Biochem. Zt., **103**, 178 (1920).

the reaction of the solution to the acidic side according to the salts and their concentrations, in the following order:

$$BaCl_2 < CaCl_2 < MgCl_2 < CoCl_2 < NiCl_2 < CuCl_2.$$

Hence, we find the difference in the powers of cations in the chlorides, which strengthen the hydrogen ion activity in the solution, in the order $Ba^{\cdot\cdot} < Ca^{\cdot\cdot} < Mg^{\cdot\cdot} < Co^{\cdot\cdot} < Ni^{\cdot\cdot} < Cu^{\cdot\cdot}$. Above all, such a metal ion as $Co^{\cdot\cdot}$, $Ni^{\cdot\cdot}$, or $Cu^{\cdot\cdot}$ in chloride acts a powerful influence on the reaction of the solution. The precipitability of the rice-glutelin changes also according to the addition of these salts. In the case of $CaCl_2$ or $MgCl_2$, if its concentration is 0.005 n, the amount of flocculated protein decreases. But if the concentration of the salt increase to 0.02 n, the amount of precipitated protein will increase according to the salt-concentration. On the contrary, if the salt-concentration increase over

Fig. 4. a.

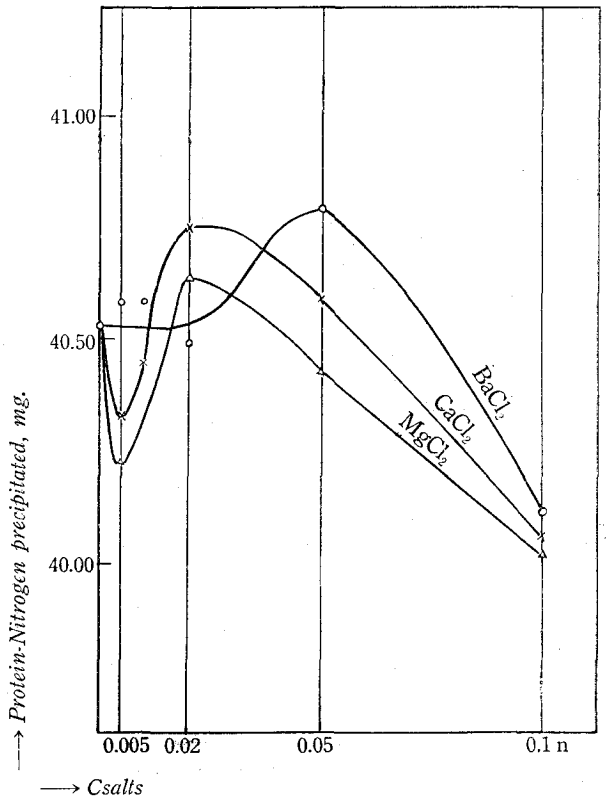
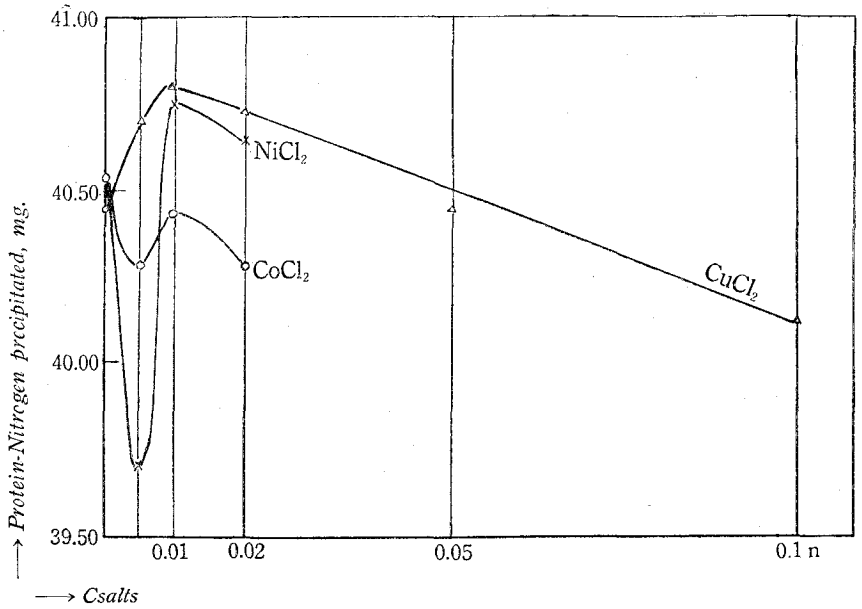


Fig. 4. b.



0.02 n, the amount of the precipitated protein will again decrease. In the case of $BaCl_2$, the precipitability of the rice-glutelin decreases according to the $BaCl_2$-concentration until it increases to 0.02 n. However, if the $BaCl_2$-concentration increase over 0.02 n, the amount of flocculated rice-glutelin again will increase. Also, if the $BaCl_2$-concentration increase more than 0.05 n, the precipitability of the protein will decrease. In the case of $CoCl_2$, $NiCl_2$ or $CuCl_2$, the influence on the precipitability of the protein is more clearly seen. If these salts be added to the solution even in the concentration of 0.005 n, the precipitability of the protein will immediately decrease. But if the salt-concentration increase more than 0.005 n, the amount of the precipitated protein will increase according to the salt-concentration until it reaches 0.01 n. However, if the salt-concentration increase more than 0.01 n, the precipitability of protein will again decrease.

Experiment c.

Here we used trivalent cations. The applied salts were $FeCl_3$ and $AlCl_3$. The plan of the experiment was identical with that described

in Experiment a. However, the influence of these salts is extraordinarily strong, hence we began our study from the concentration 1×10^{-4} n.

The experimental results are shown in Table 4 and Figure 5 and 6.

Fig. 5.

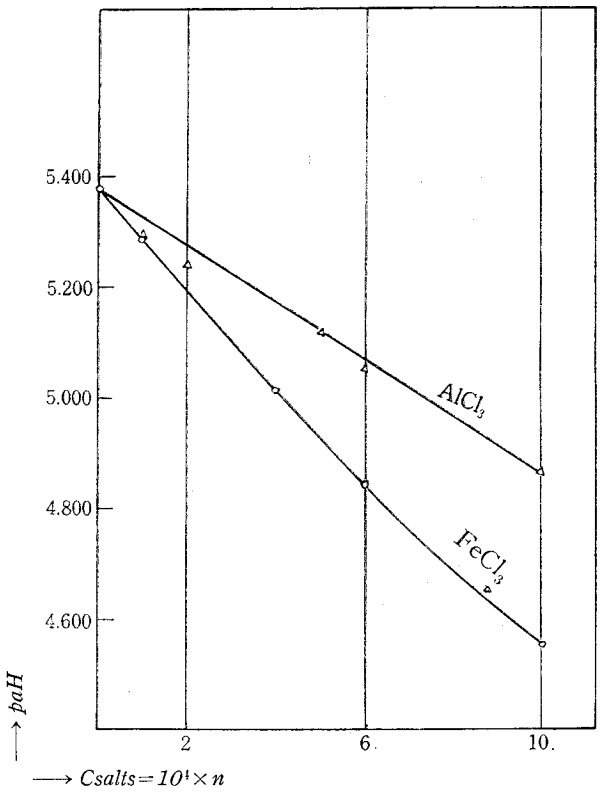


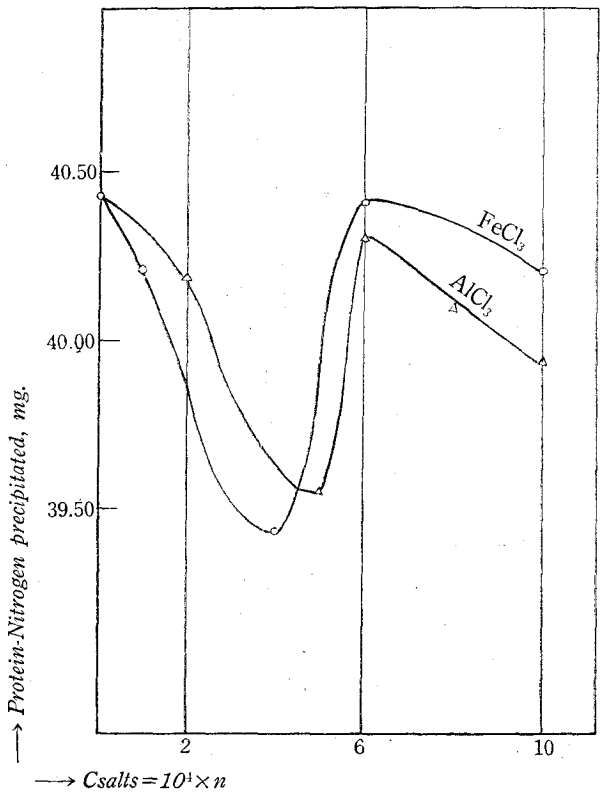
Table 4. a.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{FeCl_3} n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.001	40.23	4.551
2	,,	,,	,,	0.0006	40.43	4.844
3	,,	,,	,,	0.0004	39.44	5.013
4	,,	,,	,,	0.0001	40.23	5.286
5	,,	,,	,,	0	40.44	5.377

Table 4. b.

No.	Total protein-N in Solution mg.	$C_{\frac{NaCH_3COO}{n}}$	$C_{\frac{HCH_3COO}{n}}$	$C_{\frac{AlCl_3}{n}}$	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.001	39.96	4.867
2	,,	,,	,,	0.0008	40.12	4.961
3	,,	,,	,,	0.0006	40.23	5.054
4	,,	,,	,,	0.0005	39.55	5.115
5	,,	,,	,,	0.0002	40.02	5.245
6	,,	,,	,,	0.0001	40.54	5.288

Fig. 6.



According to the above results we see that the addition of $FeCl_3$ or $AlCl_3$ to the solution, the reaction of which is apparently iso-electric for the rice-glutelin, carries the reaction of the solution remarkably to the acidic side in the order $AlCl_3 < FeCl_3$, though the salt-concentration is so extremely diluted as $1 \times 10^{-4} - 10 \times 10^{-4}$ n. The influences of these salts are far greater than those of $CuCl_2$ and $NiCl_2$.

The precipitability of the rice-glutelin also changes distinctly under the presence of $AlCl_3$ or $FeCl_3$. Until its concentration increases to $4 \times 10^{-4} - 5 \times 10^{-4}$ n, the higher the concentration of the salt becomes, the greater the solubility of the rice-glutelin. However, if the concentration of $AlCl_3$ or $FeCl_3$ increase more than 6×10^{-4} n, the precipitability of the rice-glutelin will again decrease. This is the same in the case of the other chlorides.

C. Discussion and Conclusion. (1)

According to our theory,(1) the anion, derived from the salt, causes the protein-cation, and inversely the cation causes the protein-anion. The smaller the difference of the protein-ionizeing-forces of the two ions, the more the protein ion will be reduced to the non-iogenic form. Of course, the hydrogen ion activity plays an important role in the reduction.

Experiment "a" deals with the influences of RbCl, KCl, NaCl and LiCl on the iso-electric behavior of the rice-glutelin in the acetate-acetic acid solution. The results show that until the salt-concentration increases to a certain degree (for NaCl and LiCl 0.75 n, and for RbCl and KCl 0.5 n) the higher the concentration becomes, and the less the precipitability of the rice-glutelin. However, if the salt-concentration increase more than the above concentration, the amount of the precipitated protein will again increase. Especially in the case of RbCl, when its concentration increases to 1.0 n, the precipitability of the rice-glutelin increases more than in the case without RbCl. The above phenomena may be explained by our theory, that is, the higher the salt-concentration increases to the above mentioned limit, the greater the difference in protein-ionizeing power between the alkaline-cations and chlorine-anions derived from the salts. Hence, the precipitability of the protein decreases as much as correspondent with the difference of forces. This is one of the principles on which the so-called solvent action of the salt on

(1) Kondo K. and Hayashi T., Memoirs of Coll, Agri., Kyoto Imp. Univ. No. 2. Art II and III. (1926)

the protein is based. However, if the salt-concentration increase over the above mentioned limit, the salt or one of its components may combine with protein and result in a further precipitation. This precipitation may be due to the formation of a complex-compound combining the protein and one component of the salt. This may be an original principle of the so-called precipitating action of the salt on the protein. Then, the protein-precipitate here obtained may consist of the mixture of the non-iogenic rice-glutelin and the complex-salt-formed rice-glutelin. Therefore, we can not assume the reaction of the solution, in which, though the precipitability of the protein increased to a maximum point, to be an iso-electric reaction of the protein, when the salt-concentration is high. Because, the protein may not precipitate in all phases of the simple non-iogenic form in such a solution. Consequently, we may say that it is impossible to determine the iso-electric point of the protein simply by finding the maximum point of the flocculation of the protein. We must first have in mind the kind and concentration of the salt in the buffer solution.

Wilson and Kern[1] found two minimum points of swelling for the gelatin in the buffer-solution, e.g. at pH value of 4.7 and of 7.7 and they assumed that the gelatin shows two iso-electric reactions at these points. However, Atkin and Douglas[2] show us that the second minimum point of swelling for gelatin, which has been shown by Wilson and Kern, is not due to the second iso-electric behavior of the gelatin, but is due to the salt in the solution. If we apply our own conclusions as previously described to the above discussion, we may agree with the proposition of the latter, and we are able to confirm unmistakably the proposition that the gelatin shows an iso-electric behavior at a single point.

As shown in the above figures, the higher the concentration of the salt becomes, in a parallel manner, the greater the activity of the hydrogen ion, but the precipitability-curves turn at a certain salt-concentration. Herewith we are able to confirm the proposition that the salt itself, as well as the hydrogen ion activity, may play a great part in the role of the dissolution and precipitation of the protein. This action of the salt is due to the cations derived from the salt, and this action of the cations is peculiar to the kind of salt and its concentration. With chlorides we may arrange these cations in the order of their

(1) Wilson J.A. and Kern E.J., Journ. Amer. Chem. Soc., **44**, 2633 (1922).
(2) Atkin and Douglas: D.J. Lloyd; Chem. Protein and its Economical Application. London. 196 (1926).

mobilities : $Rb^{\cdot} < K^{\cdot} < Na^{\cdot} < Li^{\cdot}$. Multivalent cations may also be arranged in the order of their mobilities. The specific action of the ions on the flocculation and dissolution of the protein is confirmed also by the following single example. The addition of LiCl to the solution shifts remarkably the reaction of the solution to the acidic side, but, on the contrary, its influence upon the precipitability of the protein is almost the same as that of NaCl.

J. Loeb(1) states the proposition that the influence of an acid, base or neutral salt on the physico-chemical properties of the protein solution is explained only by the valency rule. However, in this field, according to the phenomena alluded to, we must bear in mind the specific actions of ions as well as their valence. Therefore we may agree with W. D. Bancroft,(2) but not with J. Loeb, for the former sustains Hofmeister's rule which the latter does not.

According to the results of Experiment "b" the presence of the chlorides of alkaline-earths or other metals carries the reaction of the acetic acid-acetate solution with the rice-glutelin to the acidic side more strongly than the presence of the alkaline-chloride. With $CuCl_2$ especially we note a very strong influence. Concerning the influence of the salt on the iso-electric rice-glutelin, while the salt-concentration is diluted, (for $BaCl_2$ to 0.02 n, and for the other chlorides to 0.005 n) the rice-glutelin dissolves according to the difference of the protein-ionizeing powers of the ions derived from the added salt. Of course, the increase of the hydrogen ion activity may also influence this dissolution. However, if the salt-concentration increase more than the above limit, the protein may combine with the cations derived from the salt and form an insoluble complex-compound. Herewith the precipitability of the rice-glutelin increases, notwithstanding the fact that the hydrogen ion activity may be higher. On the contrary, if the salt-concentration increase, (for $CaCl_2$ and $MgCl_2$ over 0.02 n, for $BaCl_2$ over 0.05 n, for $CuCl_2$, $CoCl_2$ and $NiCl_2$ over 0.01 n) the amounts of the precipitated protein will decrease gradually in accordance with the salt-concentration. This may be due to the formation of a soluble or dissociable complex-compound formed of the protein and the cations derived from the salt. This formation may perhaps be caused by the increase of the salt-concentration and the accompanying increased activity of the hydrogen ion.

(1) Loeb J: Proteins and the Theory of Colloidal Behavior. 13, 65, 88 (1922) New-York.

(2) Bancroft W.D.: Applied Colloid Chemistry. 255 (1921) New-York.

From other data it may be inferred that a protein forms a complex-salt with one component of an inorganic salt. E. J. Cohn[1] shows us that the solubility of the protein in the salt-solution follows the equation formulated by Brønsted. The solubility of casein increases, when a small amount of salt added. On the contrary, the solubility decreases on the addition of larger amounts of salt. After Cohn, this also can be explained by Brønsted's equation if we assume that casein forms a complex-compound with the salt. Moreover, Cohn believes that, the protein which flocculates out in the concentrated salt solution can be explained theoretically by Brønsted's equation. This is done, of course, under the assumption that the protein, completely soluble in the salt solution, forms a complex compound with the salt.

According to the data of Procter and Wilson[2] or Loeb,[3] the distribution of the ions between the inside and the outside of protein particles at equilibrium is in accordance with Donnan's theory, when the protein is dissolved in acids. However, Northrop and Kunitz[4] confirm the supposition that if to gelatin be added $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Zn^{\cdot\cdot}$ or $K^{\cdot}$ respectively, the distribution of these ions between the inside and the outside of the gelatin particles will not follow Donnan's formula. The cause is found to be that the gelatin forms a complex compound with these ions. This has been confirmed by Northrop and Kunitz after the E. M. F. determination of Zn and Cl potentials in the Gelatin-HCl-$ZnCl_2$ solution.

Greenberg and Schmidt[5] find that the transport numbers of casein in solutions of the alkaline-earth elements are abnormally high. They referred to the findings of Robertson[6] and of Adolf[7] that the conductivity of the serum-globulinates of alkaline-earths is markedly lower than a similar solution of alkalies. With data and references, Greenberg and Schmidt show that casein forms a complex salt with part of the alkaline-earth elements and the complex anions thus formed have a definite composition. According to the above examples, it is most probable that the protein combines with the salt-component, becoming a complex compound. Hence it may be reasonable that our data,

(1) Cohn, E.J., Journ. Biol. Chem., **59,** iv and vii, (1924.)
(2) Procter, H.R. and Wilson J.A: Journ. Chem. Soc., CIX. 307 (1916).
(3) Loeb J., Proteins and the Theory of Colloidal Behavior. 120 (1922) New-York.
(4) Northrop J.H. and Kunitz M., Journ. Gen. Physiol. **7.** 25 (1924-25).
(5) Greenberg D.M. and Schmidt C.L.A.; Journ. Gen. Physiol., VIII, No. 3, 271 (1926).
(6) Robertson T.B., Journ. Phys. Chem., XV, 166 (1911).
(7) Adolf M., Kolloidchem. Beihefte, XVII, 1 (1923).

described as above and as follows, are explained in the conception of the forming of the complex salt.

The experimental results obtained in Experiment "c" are also explained by the same theory, as described above. The influence of $FeCl_3$ or $AlCl_3$ is stronger than that of the chlorides of bivalent cations. Therefore, even with small amounts of these salts, the reaction of the solution shifts remarkably to the acidic side and the precipitability curve turns accordingly. The first point of turning shows us the formation of an insoluble complex compound and the second that of a soluble complex compound.

Repeatedly going over the results in Experiments "a", "b" and "c" shows us clearly that the influence of the chlorides on the behavior of rice-glutelin and the reaction of the solution becomes stronger with the increase of the valence* of cations. In other words, the higher the valence of cations, the greater the difference, between chlorine ion and cations in protein-ionizeing power. That is, the precipitability curve of the protein has already been turned at the point of lower concentration of salt with the cations of higher valence. Therefore, in the presence of salt, especially with multivalent ions, even in low concentration such a protein as rice-glutelin or casein may exhibit maximum flocculation at a hydrogen ion activity other than the iso-electric point of the protein. (Note results in Experiment "c") The same theory has been discussed by Cohn.(1) Hereupon, it is clear that the presence of the salt plays an important part in the determination of the iso-electric point of the protein.

According to the results in Experiments "b" and "c", $Cu^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$ or $Al^{\cdot\cdot\cdot}$, even in low concentration, forms a complex compound with protein. We know that $Cu^{\cdot\cdot}$ is very poisonous for organisms, expecially for algae and fungus. We know also that the presence of iron and alumina in the soil is considered as one of the sources in the formation of acid soil. The sterility of plants in such a soil may be due to inhibition of the life-process in the formation of a complex compound

* Hardy,(a) Mellanby,(b) Osborne and Harris,(c) Hopkins and Savory(d) have found that the solvent or precipitating action of salts upon globulins increases distinctly with the valence of their ions.

(a) Hardy, W.B., Jour. Physiol., XXIV, 288 (1899) and XXXIII, 251 (1905).
(b) Mellanby, J., Journ. Physiol., XXXIII, 338 (1905).
(c) Osborne, T.B. and Harris, I.F., Amer. Journ. Physiol., XIV, 151 (1905).
(d) Hopkins, F.G. and Savory, H., Journ. Physiol., XLII, 189 (1911).
(1) Cohn, E.J., Journ. Gen. Physiol., **4**, No. 6, 697 (1922).

between $Fe^{\cdots}$ or $Al^{\cdots}$ in the soil and the protoplasm-protein of the plant cell as well as to the acidification of the soil-reaction. The mechanism of the poisoning action of $Cu^{\cdot\cdot}$ or other heavy metal ions on organisms may be explained by the conception described above.

D. Experimental Results. (2)

Experiment d.

In this portion we have examined the influence of various anions on the maximum flocculation of the rice-glutelin. The anions are used

Table 5. a.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{KBr} n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.5	39.28	5.349
2	,,	,,	,,	0.2	39.44	5.357
3	,,	,,	,,	0.1	39.88	5.364
4	,,	,,	,,	0.05	39.86	5.363

Table 5. b.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_{KI} n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.5	38.58	5.397
2	,,	,,	,,	0.2	38.68	5.383
3	,,	,,	,,	0.1	39.15	5.384
4	,,	,,	,,	0.05	39.41	5.395

Table 5. c.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	$C_{K_2SO_4}$ n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.5	40.02	5.356
2	,,	,,	,,	0.2	40.17	5.360
3	,,	,,	,,	0.1	40.49	5.377
4	,,	,,	,,	0.05	40.64	5.392

in the form of potassium salt of SO_4'', CO_3'', Br' and I' as inorganic anions and citrate-, acetate-, tartarate- and oxalate-ion as organic anions. The experimental results with KI, KBr and K_2SO_4 are summarized in Table 5 and Figures 7 and 8.

These results show that the presence of KI, KBr or K_2SO_4 in the solution, the reaction of which is apparently iso-electric for the rice-glutelin, shifts the reaction a little to the acidic side. Moreover, the precipitability of the rice-glutelin decreases with salt added in the concentration of 0.05 n–0.5 n. If we compare these results with those

Fig. 7.

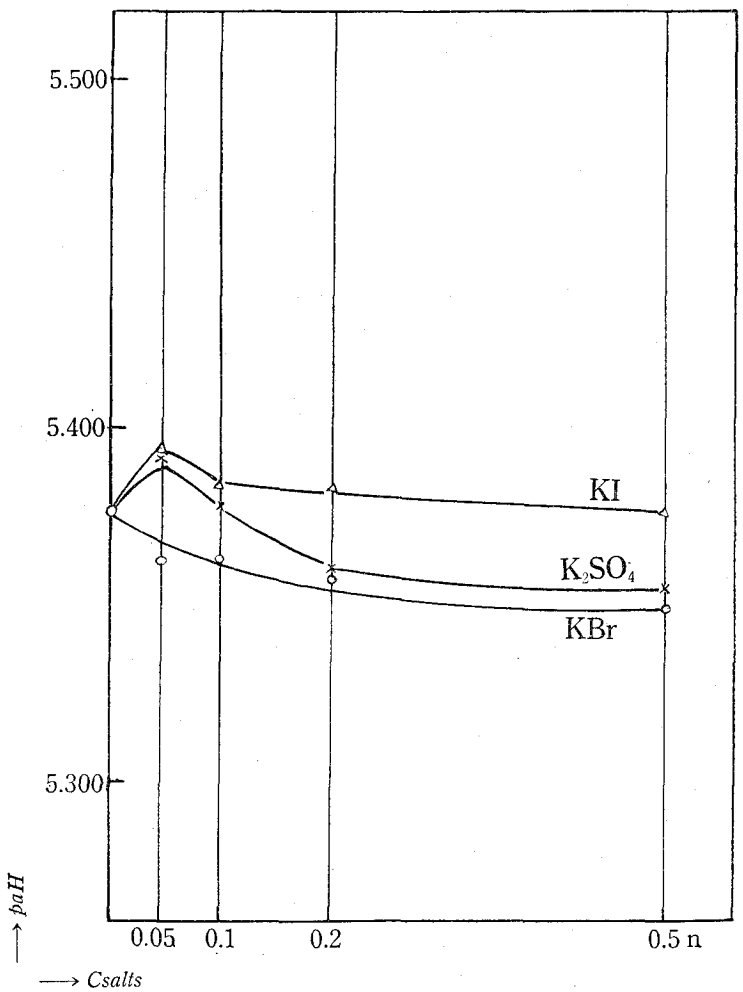


Fig. 8.

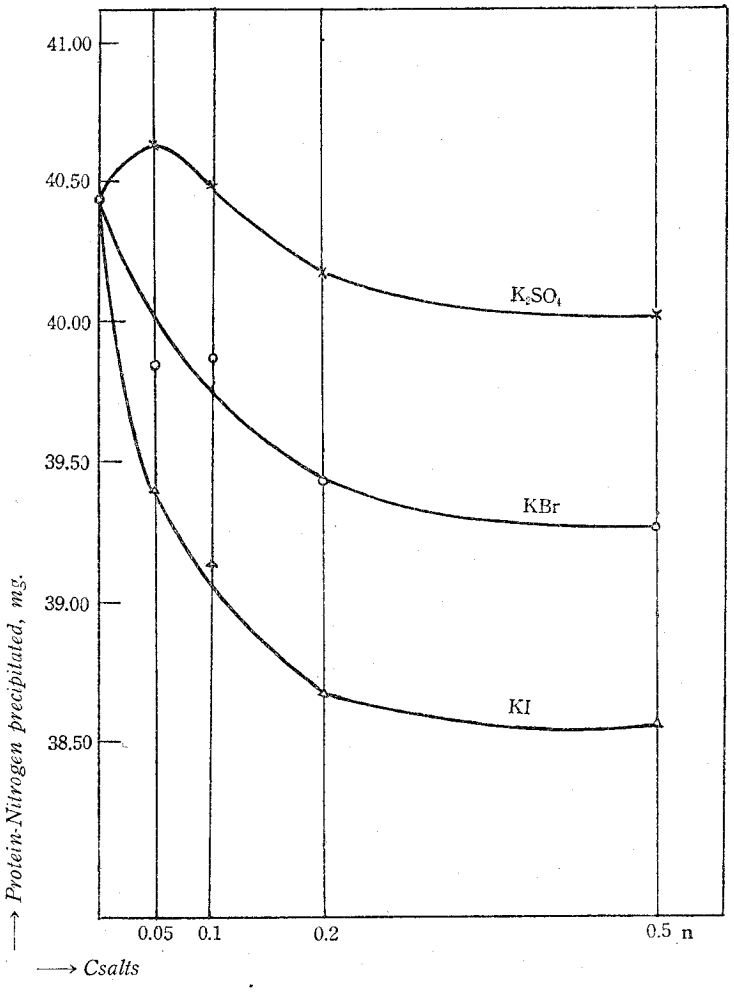


Table 6. a.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	$C_{K\text{-}Oxalate}$ n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	1.0	39.50	5.404
2	,,	,,	,,	0.4	39.40	5.393
3	,,	,,	,,	0.2	39.66	5.380
4	,,	,,	,,	0.1	39.21	5.396

Table 6. b.

No.	Total Protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_K-Tartarate n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	1.0	39.67	6.337
2	,,	,,	,,	0.4	39.57	5.910
3	,,	,,	,,	0.2	39.72	5.453
4	,,	,,	,,	0.1	39.72	5.414
5	,,	,,	,,	0.05	39.75	5.400

Table 6. c.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_K-Acetate n	Protein-N Precipitated mg,	paH
1	40.97	0.1	0.02	1.0	39.26	6.527
2	,,	,,	,,	0.5	39.16	6.188
3	,,	,,	,,	0.2	38.76	5.892
4	,,	,,	,,	0.1	39.06	5.688
5	,,	,,	,,	0.05	39.78	5.533

Table 6. d.

No.	Total protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	C_K-Citrate n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.4	37.72	6.378
2	,,	,,	,,	0.2	37.52	6.068
3	,,	,,	,,	0.1	37.76	5.837
4	,,	,,	,,	0.05	38.61	5.682

Fig. 9.

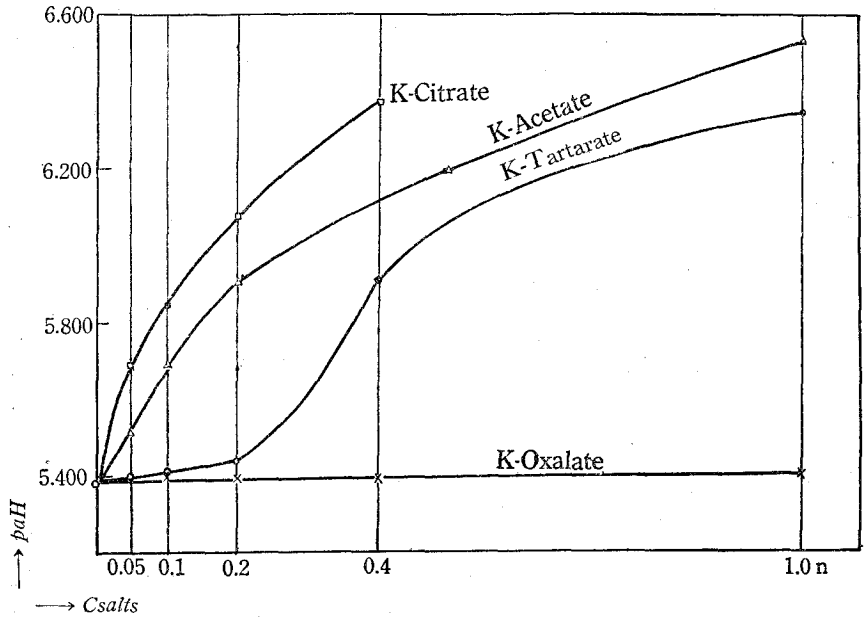
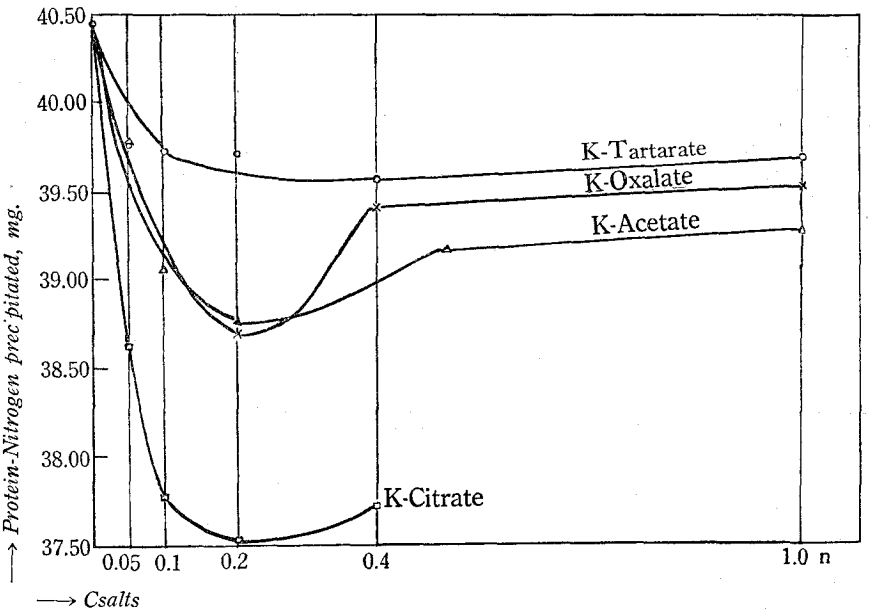

6.600
6.200
5.800
5.400
K-Citrate
K-Acetate
K-Tartarate
K-Oxalate
0.05 0.1 0.2 0.4 1.0 n
⟶ paH
⟶ Csalts

Fig. 10.


40.50
40.00
39.50
39.00
38.50
38.00
37.50
K-Tartarate
K-Oxalate
K-Acetate
K-Citrate
0.05 0.1 0.2 0.4 1.0 n
⟶ Protein-Nitrogen precipitated, mg.
⟶ Csalts

of Experiment "a", we may concluded that with potassium-salts the anions reduce the amount of precipitated protein in the order $Cl' > I' > Br' > SO_4''$.

Experiment e.

The experimental results with K-Oxalate, K-Tartarate, K-Acetate, and K-Citrate are shown in Table 6, Figures 9 and 10.

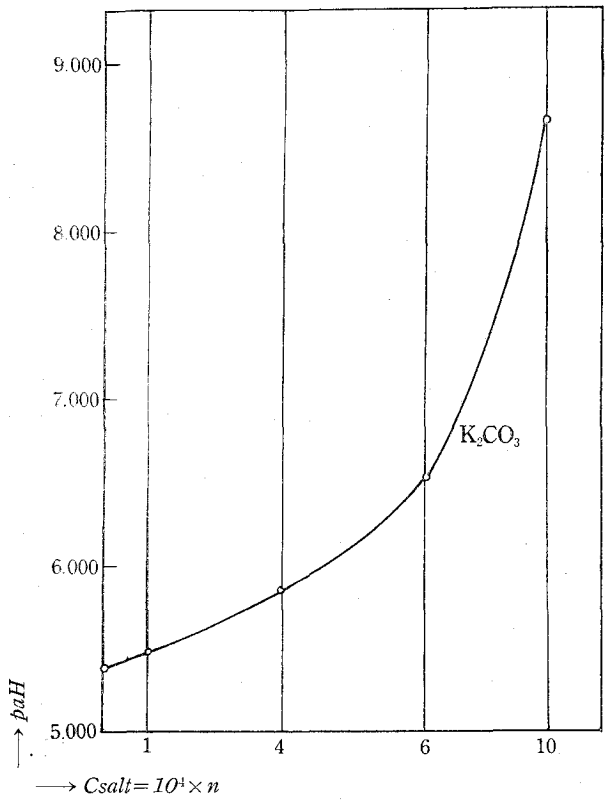
As is made clear in Table 6 and Figure 9, with potassium salts of these organic acids, the higher the concentration (0.05–1.0 n) becomes, the more the reaction of the solution shifts to the alkaline side. This is quite contrary to the phenomena with inorganic potassium salt. With potassium salts, the organic anions may carry the reaction of the solution to the alkaline side in the order Oxalate < Tartarate < Acetate < Citrate. But the influence of K-Oxalate on the reaction of the solution is almost negligible.

Figure 10 shows the influence of these salts upon the precipitability of the rice-glutelin. According to this figure, the higher the concentration of the added salt from 0.05 n to 0 2 n becomes, the greater the decrease of precipitability of the protein. However, if the salt concentration increase more than 0.2 n, the amount of precipitated protein will increase gradually, with the salt added. Moreover, if the salt is added in a concentration than 0.5 n, the precipitability of the protein will increase very gradually in accordance with the salt-concentration. The influence of these potassium salts upon the precipitability of the protein is in the order Tartarate < Oxalate < Acetate < Citrate. As described above, the potassium oxalate has a very weak influence on the reaction of the solution, but on the contrary, a somewhat strong influence on the precipitation of the protein. It is stronger than that of potassium-tartarate.

Table 7.

No.	Total Protein-N in Solution mg.	C_{NaCH_3COO} n	C_{HCH_3COO} n	$C_{K_2CO_3}$ n	Protein-N Precipitated mg.	paH
1	40.97	0.1	0.02	0.001	38.87	8.661
2	,,	,,	,,	0.0006	40.02	6.533
3	,,	,,	,,	0.0004	40.33	5.866
4	,,	,,	,,	0.0001	40.43	5.478

Fig. 11.



Experiment f.

Potassium carbonate behaves in the protein solution in the same manner as the potassium organic salts, but with a stronger influence. The results are shown in Table 7, and Figures 11 and 12.

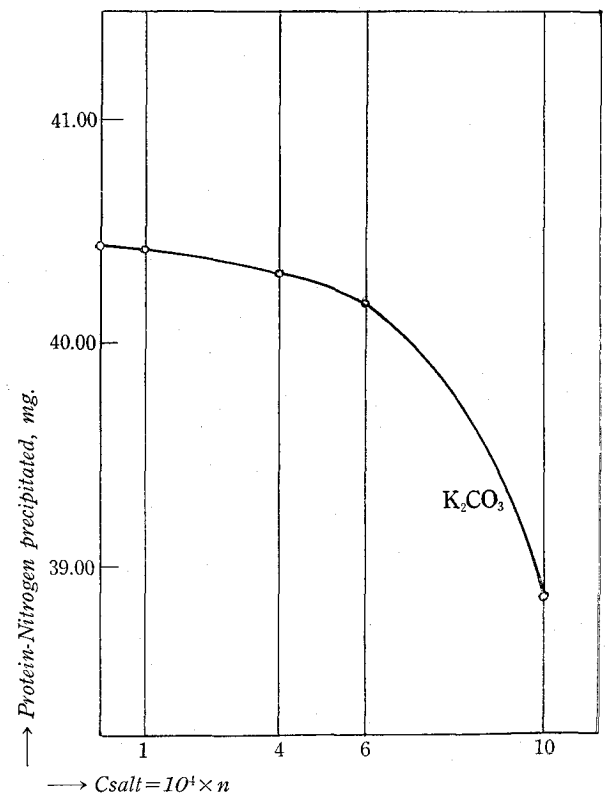
As the above table and figures show, the presence of K_2CO_3, even in low concentration, shifts distinctly the reaction of the solution, which is apparently iso-electric for the rice-glutelin, to the alkaline side according to the salt in the concentration of $1 \times 10^{-4} - 10 \times 10^{-4}$ n. With the change of the reaction of the solution, the precipitability of the protein decreases in a parallel manner. It is more remarkably noticeable than in the case of organic salt. If we add K_2CO_3 in 0.05 n to the solution as in the case of potassium organic-acid-salt, the reaction will change strongly to the alkaline side and the rice-glutelin entirely dissolves.

E. Discussion and Conclusion. (2)

The experimental results obtained in Experiment " d " are able to be explained by the same theory as applied to the results in Experiment " a ". The cations and anions derived from the KI, KBr or K_2SO_4 differ in protein ionizeing power in the order cation<anion. Hence, as shown in Figure 7, the precipitability of the protein decreases in the presence of these salts. However, if the salt-concentration increase more than 0.5 n, the protein may combine with one component of salt into the complex-salt and flocculate out as in the case of the addition of KCl or NaCl.

As we see in Experiment " e ", the presence of the potassium organic acid-salt carries the reaction of the solution to the alkaline side in accordance with the concentration (0.05–1.0 n) of the solution. This is due to the fact that these salts consist of a strong base and a

Fig. 12.



weak acid. But the addition of the potassium-oxalate, even in high concentration, does not shift the reaction of the solution, this being caused by the fact that the oxalic acid has a higher dissociation constant than the other organic acid used.

The ions derived from these salts are assumed to have the protein-ionizeing powers in the order cations > anions. Therefore, the higher the salt concentration becomes from 0.05 n to 0.2 n, the less the precipitability of the rice-glutelin. However, if the salt concentration increase more than 0.2 n, the amount of precipitated protein will increase once more. The cause of this is sought for in the formation of an insoluble complex compound between the protein molecule and the component of the added salt, as explained in the preceding discussion.

In regard to the influence of these organic acid-salts on the reaction of the solution and the precipitability of the protein, the dissociation degrees of these salts and the dissociation constants of these organic acids may play the greatest part.

The dissociation constant of the carbonic acid is very small in relation to that of the organic acids used.

Kind of Acids	Dissociation Constant* at 25°C
Carbonic acid	3.0×10^{-7}
Acetic acid	1.86×10^{-5}
Tartaric acid	9.7×10^{-4}
Citric acid	8.7×10^{-4}
Oxalic acid	3.8×10^{-2}

Hence, the presence of the potassium carbonate, even in low concentration, shifts distinctly the reaction of the solution to the alkaline side and reduces the precipitability of the protein. This is caused by the fact that a great difference in protein-ionizeing power is found, as in the order $K^{\cdot} > Co^{''}$. Though the reaction of the solution changes remarkably to the alkaline side, the precipitability curve of the protein does not turn. The reason is that the protein may not be able to form a complex-compound with $K^{\cdot}$ or $Co_3^{''}$ in such a dilute salt-solution. Herewith we are able to confirm the supposition that the change of the hydrogen ion activity may not be the principal factor in the turn-

* Landolt, Börnstein u. Roth, Physik. Chem. Tabellen. 1912.

ing of the precipitability curve or in the formation of the protein-complex compound.

F. Summary

The main studies discussed above are summarized as follows.

(1) In this work we have studied the influence of various salts on the reaction of a solution, which is apparently iso-electric for the rice-glutelin, and also investigated the behavior of the rice-glutelin.

(2) The salts employed in these studies were the chlorides and the potassium salts. The former salts were the following twelve: RbCl, KCl, NaCl, LiCl, $BaCl_2$, $CaCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$, $AlCl_3$ and $FeCl_3$. The potassium salts used were the following eight: KI, KB, K_2SO_4, K-Oxalate, K-Tartarate and K-Citrate.

With chlorides we have compared the action of the various cations, and with potassium salts the action of the various anions.

(3) The presence of chloride in the solution shifts its reaction to the acidic side according to the concentration of the salt. With the change of the reaction, the precipitability of the rice-glutelin decreases.

The principal cause of this is found to be that the cations and anions derived from the salt have protein-ionizeing power in different degrees. Of course, the hydrogen ion activity plays a part of the role in these phenomena.

(4) However, if the concentration of the chloride increase to a certain limit, the precipitability curve of the protein will turn. This limit is moveable according to the kinds of salts. The first turning of the curve is due to the formation of an insoluble complex compound, in which the protein molecule combines with the cations of the salt. After the greater increase of the salt concentration, the precipitability curve again turns. This shows that the amount of the flocculated protein again decreases. The cause of this is also found to be that the protein molecule combines with the cations of the salt into a soluble complex compound.

The formation of the complex compound between the protein molecule and alkaline earth ions or metal ions is explained by several investigations.

(5) The higher the valence of the cations becomes, the stronger the influence of the chlorides. That is, with high valency salt, even in low concentration, the precipitability curve turns and the reaction of the solution changes remarkably to the acidic side.

Hence, in the presence of $FeCl_3$, $AlCl_3$, $CuCl_2$, and alkaline earth

chlorides, even in low concentration, the protein may exhibit maximum flocculation at a hydrogen ion activity other than the iso-electric point of the protein. The precipitate here obtained consists of a mixture of the non-iogenic- and the complex-salt-form of protein. Therefore, in the determination of the iso-electric point of the protein we must bear in mind the points above discussed.

(6) Even the cations with equal valence influence the precipitability of the protein and the reaction of the solution in different degrees. They are in the order of the mobility of the ions.

(7) According to the facts described in section (4) and (6), we may say that the various ions have their own specific actions, after Hofmeister's rule, as well as behaving according to the valency rule.

(8) The poisoning action of $Cu^{\cdot\cdot}$ on the organisms and sterility of acid soil, due to the presence of iron and alumina in the soil, may be caused by the fact that an inhibition of the living cell-action will be introduced by the formation of a complex compound, in which $Cu^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$ or $Al^{\cdot\cdot\cdot}$ combines with the protoplasm-protein in a definite ratio.

(9) The influence of KI, KBr or K_2SO_4 is explained by the same theory as elucidated in the case of alkaline chloride. The action of these anions is specific in the order of their mobilities.

(10) The presence of potassium carbonate or the potassium organic acid-salt shifts the reaction of the solution to the alkaline side in accordance with the salt-concentration, and reduce the amount of the precipitated protein. This is due to the fact that the ions derived from the salts have protein-ionizeing power in the order cation>anion. And this difference occurs because of the small value of the dissociation constant inherent in the organic acids used. Hence, the degree of the influence of these organic acid ions (including the carbonate ion) is in the order of their dissociation constants.

(11) The higher the concentration of the potassium organic acid-salt becomes, the more the alkalinity of the solution, but, the precipitability curve turns over. This is due to the formation of an insoluble complex compound in the same manner as that described in Section (4).

(12) The presence of the potassium oxalate does not shift the reaction of the solution, but on the contrary, the presence of the potassium carbonate, even in low concentration, causes a remarkable shift to an alkaline side. This is explained by the fact that the dissociation constant of the oxalic acid is relatively high, and on the contrary that of the carbonic acid is very low.

(*February 14, 1928*)